Nitrite-derived nitric oxide formation following ischemia-reperfusion injury in kidney

Masami Okamoto,1,2* Koichiro Tsuchiya,3 Yasuhisa Kanematsu,1 Yuki Izawa,1 Masanori Yoshizumi,1 Susumu Kagawa,2 and Toshiaki Tamaki1* Departments of 1Pharmacology and 3Urology, University of Tokushima School of Medicine, and 2Department of Clinical Pharmacology, Graduate School of Pharmaceutical Sciences, University of Tokushima, Tokushima, Japan Submitted 4 February 2004; accepted in final form 9 September 2004

Nitrite-derived nitric oxide formation following ischemia-reperfusion injury in kidney. Am J Physiol Renal Physiol 288: F182–F187, 2005. First published September 14, 2004; doi:10.1152/ajprenal.00036.2004.—Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS), and nitrite and nitrate are believed to be waste forms of NO. We previously reported an enzyme-independent pathway of NO generation from nitrite in animal models. In this study, we show nitrite-derived NO formation in renal ischemia-reperfusion injury using electron paramagnetic resonance (EPR) spectroscopy. In this experiment, we utilized a stable isotope of [15N]nitrite as a source of nitrite to distinguish L-arginine-derived NO from [15N]nitrite-derived [15NO]. Intravenous infusion of a stable isotope of [15N]nitrite ([15NO]) facilitated the formation of Hb15NO during renal ischemia, which demonstrated that the origin of NO was nitrite. The EPR signal of Hb15NO in kidney appeared after 40 min of renal ischemia, and renal reperfusion decreased the Hb15NO level in the kidney and increased it in blood by contrast. In addition, the amount of HbNO was nitrite concentration dependent, and this formation was NOOS independent. Our findings suggest that nitrite can be an alternative source of NO in ischemic kidney and that it binds with hemoglobin and then is spread by the circulation after reperfusion.

HbNO: electron paramagnetic resonance; N1-nitro-L-arginine methyl ester

NITRIC OXIDE (NO), a free radical molecule, has numerous roles in various physiological functions such as the regulation of the cardiovascular (9, 24), immune, and nervous systems. An imbalance of NO may cause many diseases, including hypertension, atherosclerosis, diabetes, neurodegenerative disorders, and ischemia-reperfusion injury (17, 33, 46). Renal ischemia-reperfusion injury is a critical pathological condition occurring as a result of kidney transplantation (42), and NO may play an important pathophysiological role in ischemic renal injury. However, experiments carried out in vitro and in vivo using NO synthase (NOS) inhibitors have produced an apparent discrepancy in the results (15). Moreover, it is less certain whether or how NO production occurs in the pathological stages of a renal ischemia-reperfusion event in vivo.

Generally, it has been believed that NO is synthesized from L-arginine, NADPH, tetrahydrobiopterin, and molecular oxygen catalyzed by NOS. Yaqoob et al. (47) observed hypoxia-induced elevation of NO production in isolated proximal tubules using an amperometric NO sensor and reported that this NO production occurred during hypoxia. If this is true, the mechanism is not quite convincing. How does NOS produce NO in the absence of oxygen? Samouilov et al. (38) and we (44) demonstrated an enzyme-independent pathway of NO generation from nitrite in acidic conditions. Renal vascular occlusion rapidly decreases the extracellular pH in rat kidney, and the decrease in extracellular pH is maintained during ischemia (39). Moreover, Zweier et al. (52) proposed that NO can be generated in the ischemic heart by direct reduction of nitrite to NO under the acidic and highly reduced conditions and that this NO formation is not blocked by NOS inhibitors in vitro experiments. Recently, Hirabayashi (18) detected NO production from renal cortex tissue using a NO electrode; he reported that this NO production increased sharply during ischemia and that it was independent of L-arginine administration in rats. These reports may imply that NO is produced in a NOS-independent manner under ischemic conditions and that nitrite is a candidate for the source of NO. However, it is not clear whether nitrite is responsible for the NO production in the ischemic kidney.

In the circulation, NO exists as a relatively stable hemoglobin (Hb)-NO adduct (HbNO) (35), which means that the amount of HbNO may reflect blood NO concentration. The electron paramagnetic resonance (EPR) spectrum of HbNO has a distinct three-line (Hb15NO) or two-line (Hb14NO) structure depending on the nucleus spin number of the nitrogen atom (14N = 1, and 15N = 1/2, respectively), and hence Hb is used as a natural spin-trapping agent for NO (26). In a previous report, we established an EPR subtraction method based on the subtraction of the EPR spectrum of HbNO-depleted whole blood from that of each sample that enables us to estimate the physiological concentration of HbNO (23).

The aim of the present study was to detect NO newly produced from nitrite in rat kidney during and after in vivo renal ischemia induced by renal artery and vein occlusion using the EPR subtraction method (23).

MATERIALS AND METHODS

Chemicals. Sodium nitrite (NaNO2) was obtained from Wako (Tokyo, Japan). The stable isotope of sodium nitrite (Na15NO2) was from Cambridge Isotope Laboratories (Andover, MA). N-nitro-L-arginine methyl ester (L-NAME) was from Nacalai Tesque (Kyoto, Japan). Other chemicals were of analytic grade.

Animals. Male Sprague-Dawley rats (12 wk old, weighing 350–400 g) were obtained from Japan SLC (Shizuoka, Japan) and kept in

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* M. Okamoto and K. Tsuchiya contributed equally to this work.

Address for reprint requests and other correspondence: T. Tamaki, Dept. of Pharmacology, Univ. of Tokushima School of Medicine, 3–18-15 Kuramoto, Tokushima 770-8503, Japan (E-mail: tamaki@basic.med.tokushima-u.ac.jp).
plastic cages at a controlled temperature (25°C) under controlled lighting conditions (12:12-h light-dark cycle). The animals were fed a commercial diet and had access to tap water ad libitum until the day of the experiments. All animal care and treatments were conducted in accordance with the guidelines of the animal use and care committee of the University of Tokushima.

Animal experiments. Rats were anesthetized with pentobarbital sodium (40 mg/kg body wt ip). The femoral vein and artery were cannulated with polyethylene catheters for administration of nitrite and the recording of systolic blood pressure (SBP), respectively. Nitrite (0.3 or 3 μmol/kg) was infused using a syringe pump (model 100, KD Scientific) at a rate of 1.2 mL/h. SBP was measured using an Amplifier Case 7746 (NEC San-ei Instruments, Tokyo, Japan) equipped with a pressure transducer and recorded using a WT-6856 (Nihon Koden, Tokyo, Japan). After nitrite administration, rats were subjected to left renal ischemia by occlusion of the renal artery and vein for 40 min, followed by reperfusion for 0 (ischemia only), 1, 5, or 30 min. During the experiments, rats were kept at 37°C with the use of a water jacket. After reperfusion, venous blood was collected from the vena cava with a 1-mL plastic syringe, and a left nephrectomy was performed. The kidney tissue was transferred to a 5-cm length of EPR quartz tube (Labotech, Tokyo, Japan) by puncturing. Obtained samples were stored in liquid nitrogen until the EPR measurement.

EPR measurement and data processing. All EPR measurements were carried out in liquid nitrogen. The frozen sample was directly transferred into a liquid nitrogen-filled quartz finger Dewar, which was placed in the cavity of the EPR measurement device. A JES TE 300 ESR spectrometer (JEOL, Tokyo, Japan) with an ES-UCX2 cavity (JEOL) was utilized to collect EPR spectra at the X-band (9.5 GHz). Each sample was measured four times and normalized using ESPRIT 432 software (JEOL) to improve the signal-to-noise ratio. Typical EPR conditions were as follows: power, 20 mW; frequency, 9.045 GHz; field, 3,200 ± 250 G; mod width, 6.3 G; sweep time, 60 min; time constant, 1 s; temperature, −196°C; and amplitude, 250. Spectra were stored on an IBM personal computer for analysis. The HbNO signal was obtained by subtracting the EPR spectrum of blood and kidney tissue of control rats without any further treatment from that of each sample as described previously to reveal the EPR signal of HbNO, and we compared the EPR signal intensity of HbNO by using the peak height of the first signal of a three-line hyperfine as described previously (23). EPRMAIN computer software obtained from the National Institute of Environmental Health Sciences (http://epr.niehs.nih.gov/pest.html) was used to accomplish this calculation.

RESULTS

HbNO formation from nitrite under renal ischemia. First, we investigated whether nitrite is reduced to NO during ischemia in the kidney. We monitored the HbNO formation as an index of NO in vivo by the EPR subtraction method (23). We adopted a stable isotope of nitrite ([15N]nitrite) because the Hb[15]NO EPR signal is different from the Hb[14]NO EPR signal, as indicated in Figs. 1 and 2, and using [15N]nitrite enabled us to clarify whether the source of NO is nitrite. When 3 μmol/kg of [15N]nitrite were intravenously injected into rats, unidentified EPR signals were observed in the kidney (Fig. 1A), and weak Hb[15]NO-derived EPR signals [A = 25 G (52)] appeared in the blood (Fig. 1D) in sham-operated rats. When rats were subjected to 40-min ischemia after [15N]nitrite treatment, how-

![Fig. 1](http://ajprenal.physiology.org/) Representative electron paramagnetic resonance (EPR) spectra of the kidney and blood in rats treated with [15N]nitrite followed by left renal artery and vein occlusion. [15N] has a nuclear spin of 1/2, so doublet hyperfine splitting will be observed in the EPR spectra of the Hb[15]NO complex instead of the triplet splitting observed for the natural abundance of the [14N] isotope, which has a nuclear spin of 1. A and D: rats were subject to [15N]nitrite infusion (3 μmol/kg) from the femoral vein, then provided a sham operation without renal ischemia. B and E: original EPR spectra of the kidney and blood in rats treated with saline without ischemia-reperfusion. B’ and E’: original EPR spectra of kidney and blood in [14N]nitrite treated rats followed by 40-min ischemia. B’-B and E’-E: subtraction of B from B’ and of E from E’, respectively. C and F: 40-min ischemia followed by 1-min reperfusion. The up arrow in the figure indicates the position of doublet splitting of Hb[15]NO species derived from [15N]nitrite; its constant was calculated to be 25 G. □: position of free radicals (g = 2.0); ●: position of Mn2+ (g = 2.15, 2.10, 2.03, 1.98, and 1.92 are observed in the figure, although the 6th peak is out of spectral range); ▲: position of the low-spin ferric cytochromes (g = 1.92) and iron-sulfur complex; △: position of ceruloplasmin (g = 2.06). The differences of background lines are resulting from the nitrogen bubbling during measurement. Each HbNO signal was obtained by subtracting the EPR spectrum of blood and kidney tissue of control rats from that of the sample as described previously (23). Spectral conditions were the same as the typical EPR conditions described in MATERIALS AND METHODS except that the sweep time = 15 min and the time constant = 0.3 s.
ever, marked $^{15}$NO formation was observed with the appearance of a large doublet Hb$^{15}$NO signal in the kidney (Fig. 1B’-B). On the other hand, the Hb$^{15}$NO signal in the blood after 40-min ischemia was the same as that in sham-operated rats (Fig. 1D). At 40-min ischemia and 1-min reperfusion in the kidney of the $^{15}$NO$_2$-administered rat, the Hb$^{15}$NO signal in the kidney was decreased (Fig. 1C) compared with that of a 40-min ischemia rat (Fig. 1B’-B), and circulating Hb$^{15}$NO was increased instead (Fig. 1F). Of note, several EPR signals were observed in the control kidney (Fig. 1B) and blood (Fig. 1E). Large peaks at $g = 1.92$ belong to low-spin ferric cytochromes and reduced iron-sulfur complex (40). Five peaks at $g = 2.15, 2.10, 2.04, 1.98,$ and $1.92$ are assigned as manganese(II) (the sixth peak was out of present magnetic field), and the broad signal at $g = 2.0$ and $g = 2.06$ are free radicals and ceruloplasmin, respectively (41).

In matched experiments performed with the addition of the natural abundance of 3 μmol/kg $^{14}$NO$_2$, a large Hb$^{14}$NO-derived NO triplet signal [$A_z = 18$ G (52)] was observed in both the kidney and blood after 40-min ischemia followed by 1-min reperfusion (Fig. 2, B and D). In addition, the EPR signal intensity of Hb$^{14}$NO was a function of the added $[^{14}$N]nitrite concentration. The intensity of the HbNO signals at 40-min ischemia plus 1-min reperfusion in both the kidney and blood of rats treated with 3 μmol/kg nitrite was almost four times larger than those of rats treated with 0.3 μmol/kg nitrite in both the kidney and blood (Fig. 2). These data demonstrated that at least a fraction of exogenously administered nitrite could be a source of NO during renal ischemia.

**Reperfusion time and HbNO intensity.** Next, we examined whether the HbNO produced in ischemic kidney remains in the kidney or leaves it following reperfusion. As shown in Fig. 3A,
a large EPR signal of Hb\(^{14}\)NO was observed in the ischemic kidney when the rat was treated with \([^{14}\text{N}]\)nitrite (3 \(\mu\)mol/kg) following ischemia for 40 min. The signal intensity of the Hb\(^{14}\)NO in the kidney was gradually decreased with reperfusion time (Fig. 3, B–D). On the contrary, the EPR signal intensity of Hb\(^{14}\)NO in blood was slight during ischemia (Fig. 3E), it was augmented by reperfusion (Fig. 3F), and then it decreased with reperfusion time (Fig. 3, F–H).

**Effect of \(l\)-NAME and allopurinol on HbNO formation in ischemic kidney.** To evaluate the contribution of NOS and xanthine oxidase to NO generation from nitrite, the effects of \(l\)-NAME and allopurinol were examined in rats by 40-min occlusion. Rats were orally administered \(l\)-NAME (1 g/l drinking water) for 1 wk to induce NOS dysfunction rats (4, 31) or were intravenously treated with allopurinol (4 mg/kg) (50), then received intravenous injections of \([^{15}\text{N}]\)nitrite or \([^{14}\text{N}]\)nitrite (3 \(\mu\)mol/kg) followed by 40-min ischemia. As shown in Fig. 4, A and B, \(l\)-NAME treatment did not affect the Hb\(^{15}\)NO formation in the ischemic kidney of \([^{15}\text{N}]\)nitrite-treated rats. On the other hand, allopurinol treatment lowered the EPR signal of Hb\(^{14}\)NO (Fig. 4D) compared with the allopurinol-free control (Fig. 4C).

**DISCUSSION**

It has been generally accepted that NO is produced by NOS catalyzation of the oxygen-, tetrahydrobiopterin-, and NADPH-dependent oxidation of \(l\)-arginine (3). Nitrite and nitrate are believed to be waste forms of NO. However, we have demonstrated that nitrite can be an alternative source of NO during renal ischemic insult in in vivo rat experiments.

In the kidney, NO is mainly released by both constitutive and inducible isoforms of NOS, and it plays an important role in normal renal homeostasis (45). NO antagonizes the vasoconstrictive effect of angiotensin II on the afferent arterioles (20, 48) and helps to maintain glomerular filtration rate, renal plasma flow (16), and sodium regulation. NO may also play an important pathophysiological role during renal ischemia (15), but the precise role of NO in renal ischemia-reperfusion injury is not clear. It was reported that NOS activity in isolated proximal tubule cells was enhanced by 15 min of hypoxia (\(P_2\): 20–40 mmHg) with an increase of nitrite/nitrate (NOx) formation and that \(l\)-NAME treatment suppressed NOx formation in the cells (49). In addition, constitutive NOS mRNA is enhanced during hypoxia (19), iNOS mRNA is constitutively present in proximal tubules, and upregulation of iNOS occurs through rapid mechanisms independent of de novo synthesis (29). However, Hirabayashi (18), using an NO electrode, determined that NO production in renal cortical tissue increased sharply during ischemia and that this NO production was independent of \(l\)-arginine administration in rats. Furthermore, it is unlikely that NOS forms NO under ischemic condition for the following reasons: 1) molecular oxygen, an essential component for NO biosynthesis by NOS, is deficient; and 2) ischemia results in a decrease in tissue pH value (38, 39), which lowers NOS activity (10).

Zweier et al. (51) suggested that NO can be generated in tissue by either direct disproportionation or reduction of nitrite to NO under the acidic and highly reduced conditions that occur in cardiac ischemia in vitro experiments. It is likely that a considerable amount of nitrite in the renal circulation may serve as a source of NO during ischemia because concentrations from 0.4 \(\mu\)M (28, 36) to as high as 8.8 \(\mu\)M (1) nitrite are found in blood. However, there is no concrete evidence that nitrite is a source of NO in the event of renal ischemia. Therefore, we investigated whether nitrite can be an alternative source of NO during renal ischemia using the EPR subtraction technique with endogenous Hb (23). In this experiment, we utilized a stable isotope of \([^{15}\text{N}]\)nitrite as a source of nitrite to distinguish between endogenously produced NO (\(l\)-arginine with NOS) and \([^{15}\text{N}]\)-nitrite-derived \(15\)NO (26).

Renal ischemia increased the prominent doublet Hb\(^{15}\)NO-derived EPR signal in kidney while in blood it was the same as in the nonischemic condition. Renal reperfusion decreased the Hb\(^{15}\)NO level in the kidney and increased it in blood by contrast (Fig. 1), indicating that nitrite can be a source of NO generation during ischemia and that reperfusion transfers HbNO from the kidney to the bloodstream (Fig. 3). We measured the \(P_2\) values of venous blood sample because the shape of the EPR spectrum of HbNO is subject to modification by surrounding oxygen concentrations (25). However, no significant difference was observed among them (data not shown) (26). Further in vivo experiments showed that HbNO formation is a function of the intravenously administered nitrite
concentration (Fig. 2) and that it was independent of l-NAME treatment (Fig. 4, A and B). There is evidence that the content of NOx in kidney is reduced during ischemia (43), which may support our hypothesis that nitrite was converted to NO, then trapped by Hb. Of note, the small differences in the EPR-hyperfine structure in the low magnetic field between samples of blood and kidney may be responsible for the difference in the existence of unresolved EPR-active compounds (13).

Recently, two pathways for NO production from nitrite were proposed. One possible mechanism is an acid-derived disproportionation of nitrite to NO (38). This intermediate reacts with another nitrite ion to form dinitrogen trioxide (38), which results in the formation of NO by decomposition (Eq. 1).

\[
\text{NO}_2^- + \text{H}^+ \rightarrow \text{HNO}_2 \quad (1)
\]

The nitrous acid can exist as a resonance form, i.e., a nitrosonium ion with a hydroxide-like structure as an intermediate (2).

\[
\text{HNO}_2 \leftrightarrow \{\text{HOON}\} \quad (2)
\]

This intermediate reacts with another nitrite ion to form dinitrogen trioxide (38), which results in the formation of NO by decomposition.

\[
\{\text{HOON}\} + \text{NO}_2^- \rightarrow \text{N}_2\text{O}_3^- + \text{OH}^- \quad (3)
\]

\[
\text{N}_2\text{O}_3^- \rightarrow \text{NO} + \text{NO}_2^- \quad (4)
\]

However, it seems unlikely that acid decomposition of nitrite is a major pathway for NO formation in ischemic kidney because the pH in the ischemic kidney is reported to drop to ~6.5 (39), and this value is not sufficient to produce nitrous acid (Eq. 1) due to its low dissociation constant (4.6 × 10^{-10} M) (7). However, in our preliminary experiments, we found that acid decomposition of nitrite was enhanced by some kinds of reductants.

Another likely pathway for NO formation from nitrite is an enzyme-derived reduction pathway. Millar et al. (32) reported that xanthine oxidoreductase (XOR) can catalyze the reduction of inorganic nitrates and nitrites to NO under hypoxic conditions in the presence of NADH in in vitro experiments. This theory is based on the following reasoning: XOR is concentrated in endothelial cells (21, 37) and during ischemia, xanthine dehydrogenase (XDH) is converted to xanthine oxidase (XO) and adenine-guanine nucleotides are converted to hypoxanthine as a reducing substrate (6, 30). Furthermore, it was recently found that nitrite is reduced to NO at the molybdenum site of XOR under anaerobic conditions and that this reaction is favored at slightly acidic pH values (14). Our present data are somewhat in agreement with the latter hypothesis that XOR participates in the formation of NO from nitrite in the ischemic kidney (Fig. 4C and D) because pretreatment with allopurinol partially suppressed the formation of HbNO in the ischemic kidney of nitrite (3 μmol/kg)-treated rats. Recently, another pathway for the HbNO formation from nitrite has been proposed (11). Cosby et al. (5) reported that nitrite infusion into the human forearm brachial artery during basal conditions and following exercise caused a dose-dependent increase in blood flow and HbNO and that the increment of HbNO correlated inversely with hemoglobin oxygen saturation. Furthermore, Nagababu and co-workers (34) also reported that nitrite can be reduced by deoxyhemoglobin in red blood cells in an in vitro study, which results in the formation of Hb^{3+}NO complex and then interacts with additional deoxyhemoglobin in the vein to form HbNO. We need further experiments to clarify the mechanism of the formation of NO from nitrite in ischemic kidney.

In the present study, we first demonstrated a distinct HbNO signal in both ischemic kidney and circulating blood after reperfusion of nitrite-treated rats with the EPR subtraction method. These results suggest that the increased generation of NO during renal ischemia leads to the formation of HbNO and that it appears in the circulation by reperfusion. Recently, it was suggested that HbNO is a preserved form of NO (12), and this hypothesis may, in part, explain the temporary drop in blood pressure that occurs after reperfusion (8). Now, we will investigate the pathophysiological role of nitrite-derived NO during renal ischemia.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. K. Kirima for early contribution to this work and M. Shikishima for valuable help in the preparation of this manuscript.

REFERENCES
